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13. ABSTRACT ***(Maximum 200 Words)***

We hypothesize that one can use specific protein or peptide sequences to direct linked DNA molecules to breast cancer cells; this hypothesis is being experimentally tested.

During the period covered by this progress report, we have shown that we have conducted proof-of-concept studies to show that we can selectively target adenovirus vectors to a defined cell type of interest, using peptides derived by screening of phage display libraries. We are now proceeding with experiments aimed at targeting adenovirus vectors to breast cancer cells. We have generated molecules capable of binding with high affinity to the $\alpha_v\beta_3$ integrin ($\alpha_v\beta_3$ is an endocytosing receptor that is expressed on many breast cancers). These molecules were derived from a randomized library of mutants of the tenth domain from human fibronectin (FN3), displayed on the surface of M13 phage. The $\alpha_v\beta_3$-binding FN3 mutants are small proteins with great stability and much stronger binding affinity than short peptides. We are now examining whether these FN3 molecules can mediate DNA transfer into $\alpha_v\beta_3$-positive cells, including breast cancer cells.

The findings obtained to date have provided us with important reagents for further studies, including analyses of gene transfer into breast cancer cells, both in vitro and in vivo.

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FOREWORD

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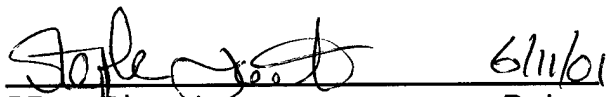

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INTRODUCTION

A number of gene delivery systems, including virally-based vectors as well as non-viral methods, are presently being explored as potential DNA delivery vehicles for gene- and immuno- therapy of breast cancer. However, currently available DNA delivery vehicles for gene therapy of breast cancer have a very wide host cell range, making it difficult to specifically target them to tumor cells. Therefore, the experiments being performed under the auspices of this grant award seek to develop innovative new approaches that will allow one to selectively target DNA molecules to breast cancer cells. The underlying hypothesis which is being explored is as follows: that one can use specific protein or peptide sequences to selectively target linked DNA molecules to breast cancer cells. This hypothesis is explored by a combination of approaches, including the use of peptide phage display libraries, recombinant adenovirus-derived gene delivery systems, and (ultimately) proof-of-concept experiments in a small animal model for breast cancer.

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- *Task 1.* Analysis of DNA delivery by adenovirus penton base proteins (months 1 - 12)
- *Task 2.* Application of phage display technology to the identification of breast cancer targeting peptides (months 1 - 15)
- *Task 3.* Analysis of DNA delivery to breast cancer cells by novel peptides (months 16-30)
- *Task 4.* Studies of DNA delivery using an *in vivo* xenograft model for breast cancer (months 25-36)

Research Accomplishments associated with the above tasks

Task 1: Experiments on the analysis of DNA delivery by adenovirus penton base proteins are summarized in the attached paper, published in Eur. J. Biochem (Bal *et al.*). This work was described in the previous annual report. Of particular relevance to Tasks 3 and 4 is the following data (reported in the manuscript by Bal *et al.*):

- ❖ Full length Ad7 penton base protein (Ad7PB) and short peptides corresponding to the integrin-binding domain from Ad7PB (Ad7PB-derived RGD peptides) were capable of mediating DNA transfer into 293 cells. However, the efficiency of this DNA transfection was approximately 100-1000x worse than that mediated by standard methods (lipofectamine).

- ❖ Ad7PB and Ad7PB-derived RGD peptides gave a very low (undetectable) rate of DNA transfer into primary cells (including dendritic cells) and were also highly inefficient in mediated DNA transfer into cell lines other than 293 cells.

- ❖ Ad7PB-mediated DNA transfer was highly variable

Implications of Task 1 findings, and modification of Timeline/Statement of Work: The observations summarized above have obligated us to explore alternative methods for DNA delivery into breast cancer cells, since it appears highly unlikely that the adenovirus penton base protein will prove suitable for *in vivo* DNA delivery. This has obligated us to extend the timeline for Task 2. At the same time, we have also made the decision to push back Tasks 3 and 4 into Year 3, since it is premature to go forward without better reagents. See below.

Modified Timeline/Statement of Work

Based on the above considerations, we have modified our timeline and statement of work, as follows:

- *Task 1.* Analysis of DNA delivery by adenovirus penton base proteins (months 1 - 12) —*as in original timeline.*
- *Task 2.* Application of phage display technology to the identification of breast cancer targeting peptides (months 1 — 24) — *extended from original timeline.*
- *Task 3.* Analysis of DNA delivery to breast cancer cells by novel peptides (months 25-36) — *delayed from original timeline.*
- *Task 4.* Studies of DNA delivery using an *in vivo* xenograft model for breast cancer (months 25-36) — *as in original timeline.*

Task 2: Experiments aimed at the identification of novel breast cancer targeting peptides that were reported in the previous annual report included the following:

- Construction of a random peptide display library in T7 bacteriophage
- Development of protocols to enrich for phage populations capable either of binding to specific cellular receptors (CD40, $\alpha_v\beta_3$ integrin), or capable of undergoing internalization in cultured human cells
- Application of these methods to breast cancer cells, and recovery of phage populations with an enhanced ability to enter breast cancer cells
- Application of these methods to a specific endocytosing cell surface receptor expressed on breast cancer cells (alphaV beta3), and recovery of phage populations with an enhanced ability to bind to this molecule *(note: in these experiments we used peptide display libraries; short peptides selected in this manner typically have only low to moderate affinity for the molecules to which they bind)*
- Construction of recombinant T7 bacteriophages which express (I) a HER-2/neu binding peptide and (II) high affinity integrin-binding proteins (snake disintegrins and recombinant derivatives thereof)

New findings are summarized on the following pages.

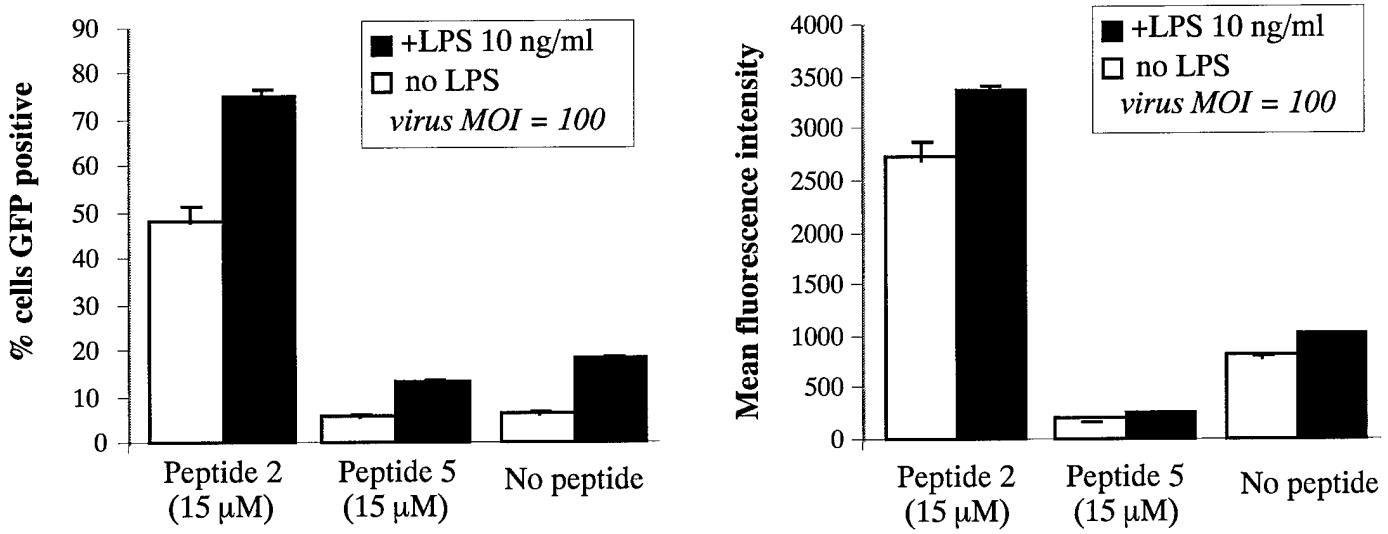
Retargeting of adenovirus vectors using phage-selected peptides. In light of our focus on adenovirus-based gene delivery technologies (Task 1), we have elected to explore the feasibility of using phage-selected peptides to retarget adenovirus vectors to cell types of interest which may express low levels of the major adenovirus receptor (the coxsackie-and-adenovirus receptor, or CAR). Cells which are deficient in CAR, or which express low levels of CAR, include many breast cancer cells. Hence, this analysis is directly relevant to improving gene delivery to breast cancer cells. Further, it offers the potential to selectively target these cells, by addressing the adenovirus vector to cell surface receptors which may be unique to, or overexpressed on, breast cancer cells (such as her2).

In pilot feasibility experiments, we have examined ability of our CD40-selected peptides (described in our previous annual report) to enhance adenovirus-mediated gene transfer into primary murine dendritic cells (DC). These cells have previously been shown to express only very low levels of the CAR receptor (5, 6), making them recalcitrant to adenovirus-mediated gene transfer (typically, one has to use very high multiplicities of infection to achieve acceptable rates of gene transfer in these cells) (1, 5, 6).

These pilot experiments were approached by synthesizing bifunctional oligopeptides, containing two distinct domains, separated by a short spacer (GGGS). Functional peptide domains were as follows: (1) a motif that binds to the adenovirus fiber protein (MH20; RAIVGFRVQWLRRYFVNGSR (2)), and (2) a phage-derived peptide selected for the ability to bind to CD40 (ATYSEFPGNLKP) or a mutated derivative of the same peptide in which the consensus CD40-binding region was replaced by alanines (ATYSEAAAALKP).

The peptides were then added to a fixed amount of an adenovirus vector expressing the jellyfish green fluorescent protein (Ad:GFP). The peptide-conjugated virus preparation was then added to the target cells, and GFP expression was quantitated 48 hours later, using flow cytometry. The results of this experiment, shown in Figure 1, revealed that phage-selected peptides can indeed enhance adenovirus infection of CD40+ dendritic cells.

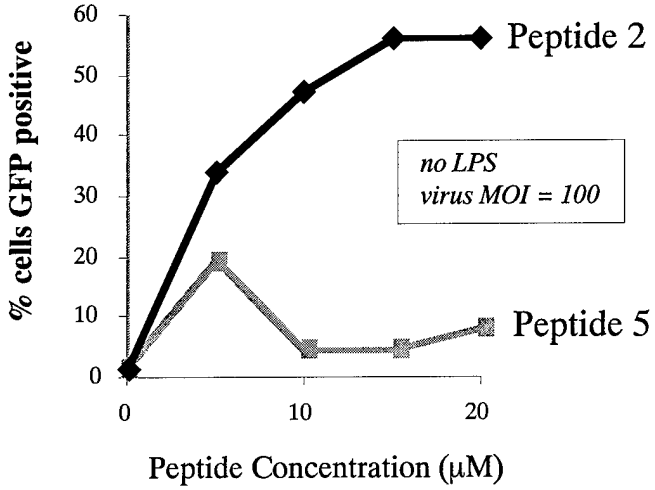
Figure 1: CD40-binding peptides can enhance adenovirus infection of dendritic cells



LEGEND LEFT: Ad:GFP was conjugated to peptide 2 (CD40-binding; ATYSEFPGNLKP) or peptide 5 (mutated peptide, lacking the consensus CD40-binding domain; ATYSEAAAALKP), or it was left untreated. A fixed inoculum of each viral preparation (standardized in terms of input PFU, measured on 293 cells) was then added to a fixed number of primary murine dendritic cells (known to be CD40-positive), and adenovirally-mediated gene transfer was then assessed 48 hours later, by measurement of the number of GFP positive cells (using flow cytometry). RIGHT: The same experiment was re-analyzed, by examining the mean fluorescence intensity in cells which were exposed to the peptide-derivatized Ad:GFP vector. It is evident that not only was there an increase in the percentage of cells which expressed GFP (left panel), but there was also an increase in the mean level of gene expression per cell (right panel),

Further studies showed that the peptide-mediated enhancement of adenovirally-mediated gene transfer into dendritic cells showed a dose-response relationship, as expected (Fig. 2).

Fig. 2: Enhanced gene transfer into dendritic cells is dependent on the dose of peptide used to retarget the adenovirus vector.

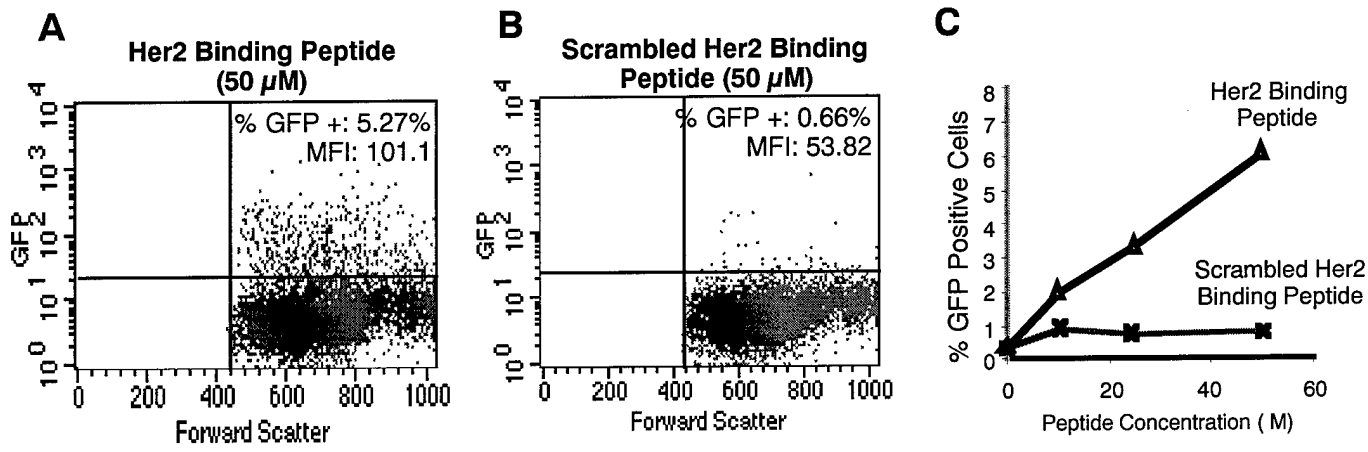


LEGEND: Methods were the same as those outlined for Fig. 1. In this case, varying amounts of the peptides (peptide 2 = CD40-binding, ATYSEFPGNLKP and peptide 5 = mutated peptide, lacking the consensus CD40-binding domain, ATYSEAAAALKP) were mixed with a fixed amount of the adenovirus vector (Ad:GFP). Gene transfer was then measured 48 hrs after exposure of primary murine dendritic cells to the vector:peptide conjugate, and is expressed as % cells which expressed GFP (as determined by flow cytometry)

The results summarized in Figures 1 and 2 provide strong support for the notion of using phage-selected peptides to target adenovirus vectors to cell types of interest. We are presently conducting experiments designed to determine if one can similarly target adenovirus vectors to her2-positive breast cancer cells. To this end, we have synthesized bifunctional peptides containing (1) a motif that binds to the adenovirus fiber protein (MH20; RAIVGFRVQWLRRYFVNGSR (2)), and (2) a peptide that has previously been shown to bind with high affinity to the her2 receptor (FCCGFYACYMDV; (4)). We are presently testing if these her2-targeted peptides can modify the ability of adenovirus vectors to infect breast cancer cells.

Preliminary studies on the use of bifunctional her2-binding peptides (RAIVGFRVQWLRRYFVNGSR- FCCGFYACYMDV) for retargeting of adenovirus vectors to her2-expressing breast cancer cells are summarized in Figure 3.

Fig. 3: Enhanced gene transfer into her2-positive breast cancer cells is dependent on the dose of peptide used to retarget the adenovirus vector.



LEGEND: Methods were the same as those outlined for Fig. 1. In this case, varying amounts of the peptides (bifunctional her2-binding peptide or a scrambled derivative of the same peptide) were mixed with a fixed amount of the adenovirus vector (Ad:GFP). Gene transfer was then measured 48 hrs after exposure of AU565 breast cancer cells to the vector:peptide conjugate, and is expressed as % cells which expressed GFP (as determined by flow cytometry). Panels A and B show results of flow cytometric analysis of GFP expression in AU565 cells exposed to the Ad:GFP —peptide conjugates (50 M peptide), while panel summarizes results from multiple experiments, performed with varying amounts of peptide.

As shown in Figure 3, the bifunctional her2-binding peptide enhanced adenovirus infection of her2-positive AU565 breast cancer cells by approximately 10-fold. We are presently examining additional cell lines, both her2-positive and her2-negative, to determine the effect of this peptide in other cells.

Future studies on the retargeted adenovirus vectors (her2-directed or $\alpha_v\beta_3$-directed) will include analyses of their ability to mediate DNA transfer into other breast cancer cells, as noted above (Task 3). Finally, retargeted adenovirus vectors with the ability to efficiently mediate DNA transfer into breast cancer cells will ultimately be examined *in vivo* (Task 4).

Development of high affinity molecules capable of specifically targeting the $\alpha_v\beta_3$ integrin. The cell adhesion receptor alpha$_v$beta$_3$ ($\alpha_v\beta_3$) integrin is of interest because of its high expression on many breast cancer cells, its role in tumor angiogenesis, and its involvement in the entry of many viruses (including adenovirus) into host cells. Previous work (reported in Year 1) using phage display methods has resulted in the identification of short $\alpha_v\beta_3$-binding peptides containing the RGD consensus motif. However, we believe that it may be possible to select for higher affinity interactions using a small protein scaffold.

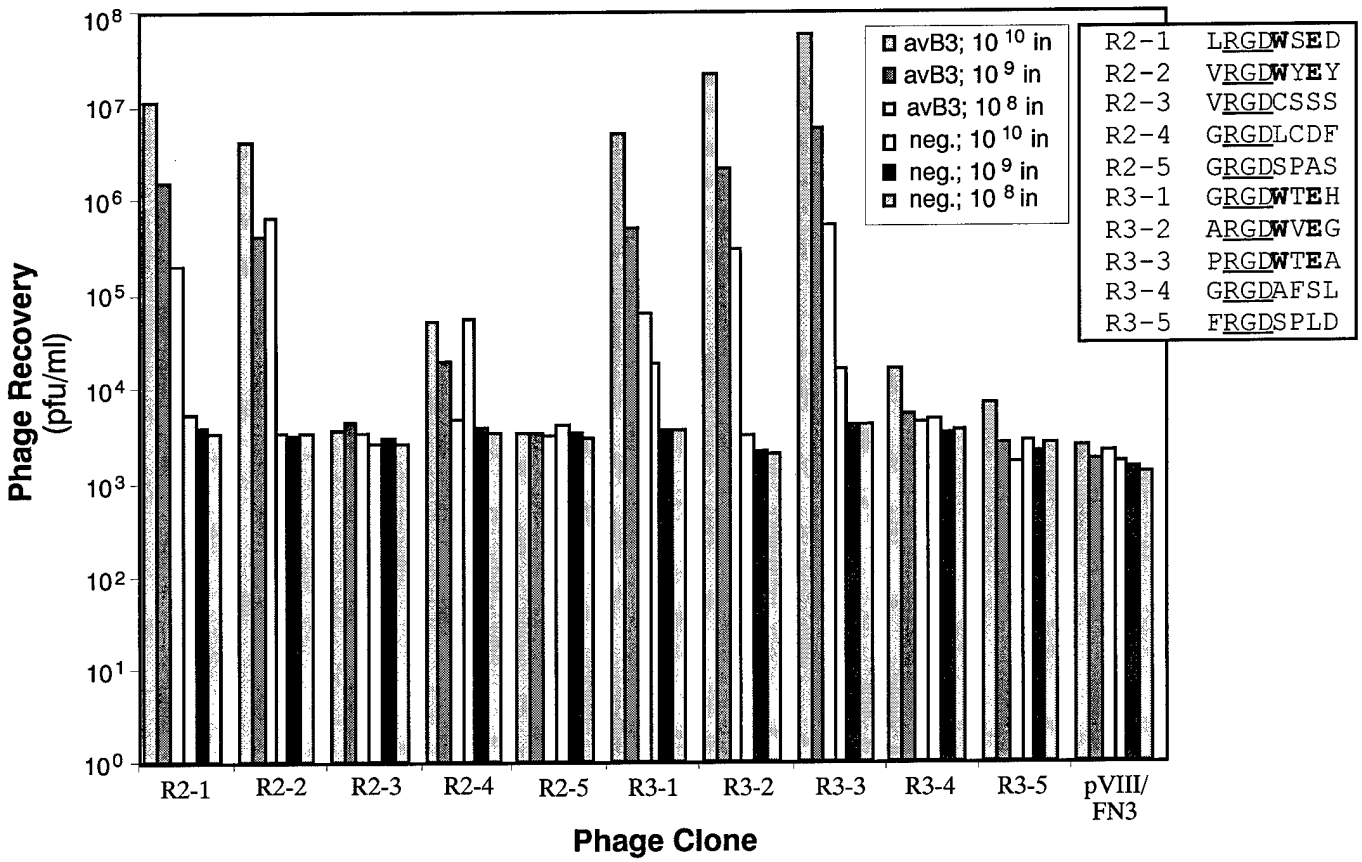
Fibronectin is a natural ligand for $\alpha_v\beta_3$, which is compromised of multiple RGD-containing subdomains. The tenth fibronectin type III domain (FN3) of human fibronectin is a extremely stable, small (< 100 amino acids), structurally defined molecule with an immunoglobulin-like structure and a loop containing the RGD sequence (3). Phage display systems for FN3 have been established, and were provided to us by Dr. Shohei Koide (3).

In order to select for FN3 sequences which exhibit an enhanced ability to bind to the $\alpha_v\beta_3$ integrin, Dr. Koide randomized the residues in the FG loop of FN3 in a library displayed on M13 bacteriophage, and then provided this library to us. Biopanning of the library against purified $\alpha_v\beta_3$ integrin (Chemicon) resulted in the selection of phage with a strongly enhanced ability to bind to $\alpha_v\beta_3$ integrin. Multiple clones from this selected phage population were subjected to DNA sequence analysis, as well as analysis of their ability to bind to $\alpha_v\beta_3$ integrin. The results are shown in Fig. 4.

The data in Fig. 4 reveal that all the clones which exhibited a strong ability to bind to the $\alpha_v\beta_3$ integrin also contained the consensus motif: RGD**W**x**E** (see clones R2-1, R2-2, R3-1, R3-2, R3-3 in Fig. 3). Thus, these data suggest that we have been able to select for a consensus motif which strongly enhances the ability of FN3 to bind to $\alpha_v\beta_3$.

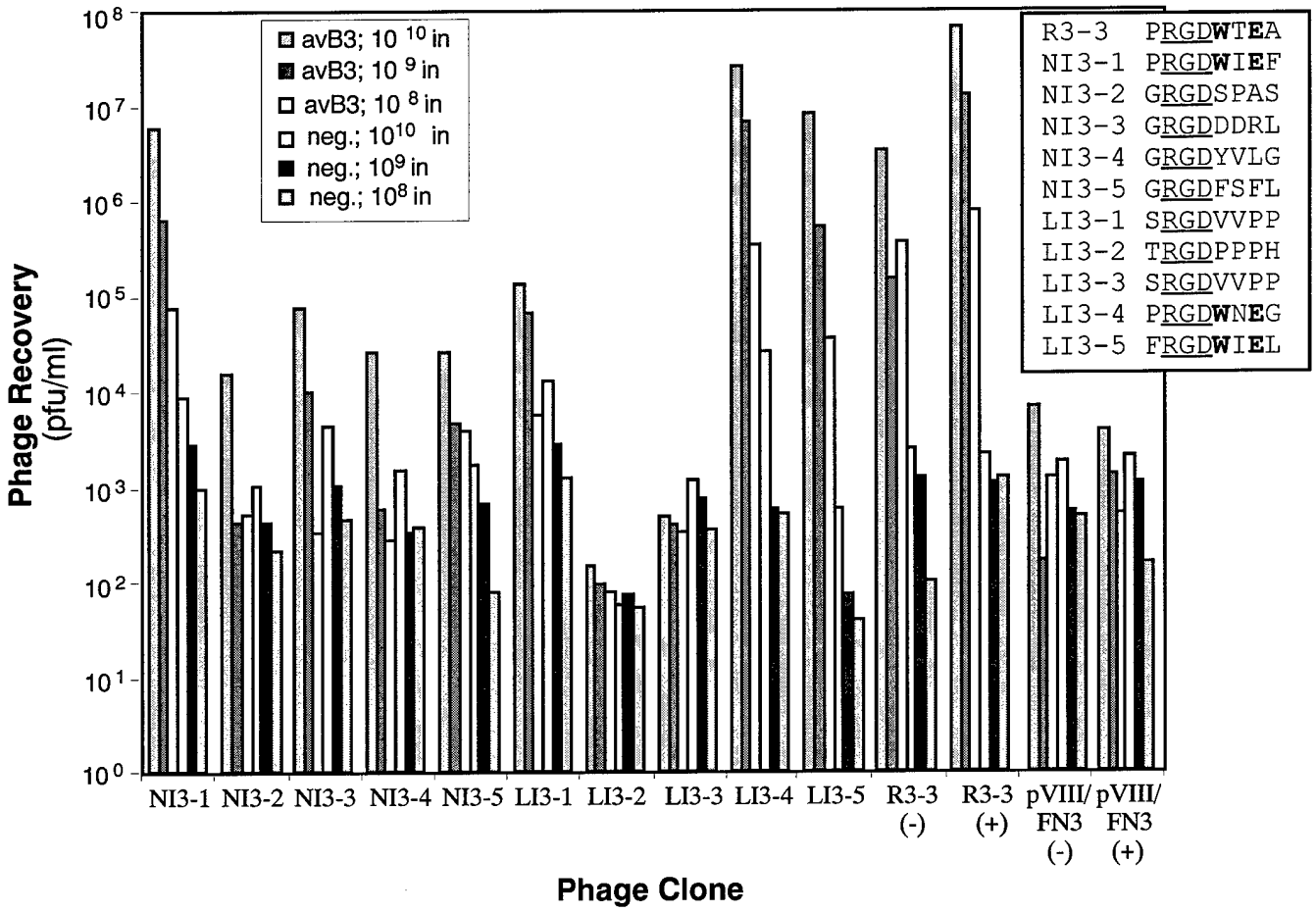
We also explored whether it might be possible to select for FN3 mutants with an even stronger affinity for $\alpha_v\beta_3$. To do, we took advantage of the IPTG-regulatable promoter system driving expression of the FN3-gene VIII fusion protein in the phagemid vector. By using either low levels of ITPG, or no IPTG at all, we were able to decrease the amount of recombinant FN3-gene VIII protein per phage particle to a very low level (estimated to be below 1 copy per phage) —thereby allowing for the possibility that we might be able to apply strong selection for high affinity interactions (and eliminating the possibility that we might select for phages which are capable of making multivalent but low affinity interactions with $\alpha_v\beta_3$). The results of this analysis are shown in Fig. 5. The most notable finding in this Figure is that we once again selected for phage which contained the same consensus motif (RGD**W**x**E**), regardless of whether we used low IPTG (LI clones) or no IPTG (NI clones) in our biopanning experiments (see clones NI3-1, LI3-4, LI3-5 in Fig. 4).

Fig. 4: Selection of FN3 mutants with an enhanced ability to bind to $\alpha_v\beta_3$ integrin.



LEGEND: The randomized FN3 phage display library (random FG loop) was propagated under conditions of full IPTG-induction (allowing for high level expression of the FN3-gene VIII fusion protein), and then biopanned against purified recombinant $\alpha_v\beta_3$ integrin (Chemicon) for three rounds. Individual clones from the second (R2-x) and third (R3-x) round of biopanning were then sequenced (inset) and they were also subjected to an analysis of their ability to bind to $\alpha_v\beta_3$ integrin (avB3) or bovine serum albumin (neg.). A range of phage dilutions (input of 10^{10}, 10^9, 10^8) were used in these experiments, and phage recovery was measured after binding and extensive washing. As shown above, clones R2-1, R2-2, R3-1, R3-2 and R3-3 all exhibited strong binding to $\alpha_v\beta_3$ integrin; the parental (unmodified) phage (pVIII/FN3) did not bind appreciably to $\alpha_v\beta_3$ integrin under the conditions used.

Fig. 5: Selection of FN3 mutants with an enhanced ability to bind to $\alpha_v\beta_3$ integrin, under conditions where expression of the FN3-gene VIII fusion protein is restricted to very low levels



LEGEND: The randomized FN3 phage display library (random FG loop) was propagated under conditions of low IPTG-induction (allowing for moderate level expression of the FN3-gene VIII fusion protein; clones designated LI), or in the complete absence of IPTG-induction (allowing for very low expression of the FN3-gene VIII fusion protein; clones designated NI). Phage populations propagated under these conditions were then subjected to biopanning against purified recombinant $\alpha_v\beta_3$ integrin (Chemicon) for three rounds. Individual clones from the third round of biopanning were then sequenced (inset) and they were also subjected to an analysis of their ability to bind to $\alpha_v\beta_3$ integrin (avB3) or bovine serum albumin (neg.). A range of phage dilutions (input of 10^{10}, 10^9, 10^8) were used in these experiments, and phage recovery was measured after binding and extensive washing. As shown above, clones NI3-1, LI3-4, LI3-5 all exhibited strong binding to $\alpha_v\beta_3$ integrin; the parental (unmodified) phage (pVIII/FN3) did not bind appreciably to $\alpha_v\beta_3$ integrin under the conditions used. Clone R3-3 (from Figure 3) is shown as a control; note that this clone bound to $\alpha_v\beta_3$ integrin whether grown under normal conditions (high IPTG induction; +) or in the absence of IPTG (-).

Ongoing analyses of the randomized FN3 library and the selected $\alpha_v\beta_3$-binding clones include:

- Selection of the FN3 library for internalization into breast cancer cells, using methods developed in Year 1.

- Purification of recombinant FN3 proteins capable of binding to $\alpha_v\beta_3$, and careful analysis of their binding affinity for $\alpha_v\beta_3$ using BiaCORE (we are presently focussing on the clones presented in Figs. 3 and 4). *Note that FN3 is readily expressed at high level in E. coli, and can be conveniently purified in a biologically active form (3).*

Future studies on the randomized FN3 phage display library include selection for clones with the ability to bind to her2, using recombinant soluble her2 (which we are presently generating).

Further studies on the selected, $\alpha_v\beta_3$-binding FN3 proteins will include analyses of their ability to mediate DNA transfer into breast cancer cells (Task 3). For this purpose, FN3 derivatives bearing an oligolysine tail will be generated (Dr. Koide has already done this, for unrelated purposes, so this is not expected to prove technically difficult).

Finally, FN3 proteins with the ability to efficiently mediate DNA transfer into breast cancer cells will ultimately be examined *in vivo* (Task 4).

Tasks 3, 4: These tasks remain to be initiated. They will be performed in Year 3.

KEY RESEARCH ACCOMPLISHMENTS

- Development of methods which establish our ability to retarget adenovirus vectors using phage-selected peptides. This will allow us to proceed with future work, in which we will retarget such vectors to breast cancer cells.

- Initiation of experiments designed at examining whether a her2-binding peptide can be used to selectively target adenovirus vectors to breast cancer cells.

- Selection of molecules capable of high affinity binding to the $\alpha_v\beta_3$ integrin, using phage display technology. These molecules are derived from the tenth domain of human fibronectin (FN3) and have enhanced physical stability and affinity, as compared to short peptides. The recombinant FN3 molecules will be examined for their ability to mediate DNA transfer in mammalian cells, via an $\alpha_v\beta_3$-dependent mechanism, and they also be tested for their ability to mediate DNA transfer into breast cancer cells.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Manuscript: H. P. Bal, J. Chroboczek, G. Schoehn, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Eur. J. Biochem. 267:6074-6081, 2000.

Abstract: J. Richards, M. Miller, A. Koide, S. Koide, S. Dewhurst. Directed evolution of fibronectin type III domain toward high affinity molecules binding to $\alpha_v\beta_3$ integrin. Presented at: "Phage Display Technologies: Directed Protein Evolution": April 2001, Boston, Mass.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training for Ms. Michelle Miller was provided (Ms. Miller is a 1999 college graduate, who has been working on this project as a laboratory technician). It is expected that Ms. Miller will attend graduate school within the next 1-2 years, to obtain her Ph.D. (personal communication from Ms. Miller), and the present research experiences should assist her in that goal. Research training for a summer undergraduate researcher, Ms. Johanna Abend, will also be provided. Ms. Abend s long-term goals remain uncertain, but likely will include graduate (Ph.D. or M.D.) school — goals which will be enhanced by her training under this award.

CONCLUSIONS

The conclusions which can be drawn from the second year of our experiments are as follows:

1. Bacterially-derived recombinant adenovirus penton base protein (ADPB) can be used to deliver linked DNA into 293 cells. However, the efficiency of this delivery process is markedly worse than that of other, commercially available transfection reagents (e.g., lipofectamine) and transfection of cell types other than 293 cells is extremely poor.

2. We have been able to develop methods which establish our ability to retarget adenovirus vectors using phage-selected peptides. These proof-of-concept studies were conducted using CD40-binding peptides (described in our previous annual report). These peptides were able to enhance adenovirally-mediated gene transfer into primary murine dendritic cells. With this technology in hand, we have now initiated experiments aimed at determining whether one can similarly target adenovirus vectors to breast cancer cells, using phage-selected peptides. We have also initiated experiments designed to test whether a her2-binding peptide can be used to selectively target adenovirus vectors to breast cancer cells.

3. We have been able to select molecules capable of high affinity binding to the $\alpha_v\beta_3$ integrin, using phage display technology. These molecules are derived from the tenth domain of human fibronectin (FN3) and have enhanced physical stability and affinity, as compared to short peptides. Mutated derivatives of FN3 which exhibit strong binding to $\alpha_v\beta_3$ are characterized by the presence of a RGD**W**x**E** consensus motif in the FG loop of the protein. These recombinant FN3 molecules will be examined for their ability to mediate DNA transfer in mammalian cells, via an $\alpha_v\beta_3$-dependent mechanism, and they also be tested for their ability to mediate DNA transfer into breast cancer cells. In addition, the randomized FN3 library and the selected $\alpha_v\beta_3$-binding clones will be selected for the ability to undergo internalization into breast cancer cells.

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APPENDICES

Manuscript: H. P. Bal, J. Chroboczek, G. Schoehn, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Eur. J. Biochem. 267:6074-6081, 2000.

Abstract: J. Richards, M. Miller, A. Koide, S. Koide, S. Dewhurst. Directed evolution of fibronectin type III domain toward high affinity molecules binding to $\alpha_v\beta_3$ integrin. Presented at: "Phage Display Technologies: Directed Protein Evolution": April 2001, Boston, Mass.

These materials are attached to this report.

APPENDIX MATERIALS

Award Number:	DAMD17-99-1-9361
TITLE:	Selective DNA Delivery to Breast Cancer Cells
PRINCIPAL INVESTIGATOR:	Stephen Dewhurst, Ph.D.
CONTRACTING ORGANIZATION:	University of Rochester Medical Center Rochester, New York 14642
REPORT DATE:	June 2001
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List of Materials Appended

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Abstract: J. Richards, M. Miller, A. Koide, S. Koide, S. Dewhurst. Directed evolution of fibronectin type III domain toward high affinity molecules binding to $\alpha_v\beta_3$ integrin. Presented at: "Phage Display Technologies: Directed Protein Evolution": April 2001, Boston, Mass.

ABSTRACT

J. Richards, M. Miller, A. Koide, S. Koide, S. Dewhurst. Directed evolution of fibronectin type III domain toward high affinity molecules binding to $\alpha_v\beta_3$ integrin. Presented at: "Phage Display Technologies: Directed Protein Evolution": April 2001, Boston, Mass.

To improve the safety and efficacy of viral vectors in vaccine and gene therapy studies, it is advantageous to exercise control over the target cell tropism of the vectors. Since phage display technology allows for the selection of receptor-specific ligands, this technique has been adapted to screening for ligands to alpha$_v$beta$_3$ integrin. Our goal is to find both high-affinity binding ligands as well as ligands which will trigger receptor internalization. The cell adhesion receptor alpha$_v$beta$_3$ ($\alpha_v\beta_3$) integrin is a target of interest because of its expression on endothelial, dendritic, and cancer cells; its role in angiogenesis; and its involvement in the entry of many viruses (including adenovirus) into host cells. Previous work using phage display methods has resulted in the identification of short $\alpha_v\beta_3$-binding peptides containing the RGD consensus motif. However, we believe that it may be possible to select for higher affinity interactions using a small protein scaffold. Fibronectin is a natural ligand for $\alpha_v\beta_3$, which is compromised of multiple domains. The tenth fibronectin type III domain (FN3) of human fibronectin is a very stable, small (< 100 amino acids), structurally defined molecule with an immunoglobulin-like structure and a loop containing the RGD sequence. We have established phage display systems for FN3 [Koide et al. (1998) J. Mol. Biol. 284:1141]. By randomizing the residues in the FG loop of FN3 in a library displayed on M13 bacteriophage, sequences binding with much higher affinity to $\alpha_v\beta_3$ were selected, all of which contain a consensus sequence. This FN3 library has also been screened for internalization into selected cell lines of interest. Future applications may include not only gene therapy/vector development but also tumor inhibition and tumor detection/visualization.

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Adenovirus type 7 penton

Purification of soluble pentamers from *Escherichia coli* and development of an integrin-dependent gene delivery system

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Adenoviral gene therapy vectors suffer from the disadvantages of toxicity and immunogenicity associated with the expression of adenoviral genes from the vector backbone. We report here an alternative strategy for gene delivery that utilizes a single component of the adenoviral type 7 capsid, the penton base (Ad7PB). The *Ad7PB* gene was sequenced and its amino-acid composition was deduced from its nucleotide sequence. The penton was expressed in *Escherichia coli* as a soluble C-terminal fusion with glutathione S-transferase (GST–Ad7PB) and was purified by single-step affinity chromatography. Both GST–Ad7PB and cleaved (GST-free) Ad7PB retained the ability to fold into pentamers as observed by electron microscopy. GST–Ad7PB was able to bind a synthetic peptide (FK20) derived from the Ad type 7 fiber and retard DNA through a polylysine chain present at the C-terminus of this linker peptide. GST–Ad7PB was an effective cell transfecting agent when assayed on 293 cells. Transfection was not dependent upon the presence of lysosomotropic agents indicating efficient endosome escape capability. Excess of an RGD-containing peptide derived from Ad7PB was able to inhibit transfection indicating specific integrin-mediated uptake of the GST–Ad7PB–FK20–DNA complexes. We propose that Ad7 pentons can be developed into integrin-specific gene delivery agents.

Keywords: adenovirus; penton base; expression; integrin; gene delivery.

The initial interaction of adenovirus (Ad) with its host cell is orchestrated by a complex of two oligomeric viral capsid proteins, penton base (PB) and fiber, which together constitute the adenoviral penton. PB protein, present at each of the 12 vertices of the icosahedral Ad capsid, associates with the N-terminal tail portion of the fiber [1,2] while the C-terminal knob of the fiber, containing the cell-binding domain, projects away from the capsid. During the process of internalization, the Ad fiber first binds to a 46-kDa transmembrane protein that functions as a high-affinity receptor for a number of adenovirus subgroups and the coxsackie B viruses [3–5]. Ad internalization then proceeds via interaction of an arginine-glycine-aspartate (RGD) sequence on the Ad PB protein with α_v integrins on the cell surface; this interaction promotes virus-receptor endocytosis [6–13]. Human adenoviruses, probably with the exception of enteric serotypes, use the vitronectin binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to promote virus internalization [6,8].

Penton base monomeric polypeptide chains assemble in a fiber-independent manner into $\approx$300 kDa homopentamers. Penton bases of some serotypes can also form higher order structures, called dodecahedra, by a combination of 12 pentamers. Penton base dodecahedra are macromolecular complexes with a molecular mass of $\approx$3600 kDa. Both penton bases and dodecahedra have been shown to interact with cell-surface integrins via RGD motifs [6,8,14,15], which suggests that they might be capable of mediating internalization in the absence of fiber.

Nonviral gene delivery methods based on the native Ad cellular uptake machinery constitute an attractive alternative strategy to Ad vector-based methods. Ad PB does not influence cellular DNA or protein synthesis when added to cell cultures [8] and may therefore minimize both the immunogenicity and toxicity problems associated with the use of Ad vectors [16–18]. The development of an integrin-specific gene delivery method may constitute a powerful means for targeting a number of vital body tissues with applications in molecular medicine.

Integrins, a superfamily of α/β heterodimeric cell surface adhesion receptors, are known to mediate cell-cell adhesion and intracellular signaling events that regulate cell survival, proliferation, and migration [19]. Endothelial cells exposed to growth factors, or those undergoing angiogenesis in tumors, wounds, or inflammatory tissue, express high levels of $\alpha_v\beta_3$ integrins [20,21]. Brooks *et al* [22]. also demonstrated a significant role for $\alpha_v\beta_3$ integrins in human angiogenesis and breast tumor growth. For most integrins, the mechanism of ligand recognition depends on one of two short peptide motifs: RGD and leucine-aspartate-valine (LDV) [23,24], both of which are present in PBs from a number of Ad serotypes, including members of the subgroup B adenoviruses (Ad3, 7 and 11).

We have previously demonstrated that dodecahedra from Ad serotype 3 can be used for gene delivery [15]. In this strategy, a linker peptide containing the first 20 amino acids of Ad3 fiber was used to combine the cell-targeting activity of the Ad3 dodecahedron with a reporter plasmid construct to

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Abbreviations: Ad, Adenovirus; PB, penton base; Ad7PB, adenovirus type 7 penton base, GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; RLU, relative light units.

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achieve intracellular delivery and expression of the reporter gene. The ternary dodecahedron-linker-DNA complex was demonstrated to enter target cells bearing integrin receptors.

Virus-cell interaction mediated through PB is known to vary by serotype [6,8,14,25]. Ad PBs show a high degree of diversity in the amino-acid sequence flanking the functionally relevant RGD tripeptide and it is possible that relative efficiencies of adenovirus PBs in mediating internalization may be a function of these differences. Ad serotype 7, another member of subgroup B adenoviruses, is known to form penton dodecahedra [26]. We therefore set out to sequence the PB gene from Ad serotype 7. Bacterial expression systems have previously been used for the expression and purification of PB from Ad serotype 2 and 12 [6,14]. However, both Ad2 PB and Ad12 PB were found to localize in *E. coli* as insoluble inclusion bodies. Our objective in pursuing this work was twofold: (a) to devise a simpler expression strategy for PB that yielded soluble protein and preserved the structural and functional integrity of the PB; and (b) to test the suitability of Ad7 penton as a gene transfer vehicle.

We describe here the sequencing, production from *E. coli* and the structural characterization of Ad7PB. The amino-acid composition of Ad7PB revealed strict conservation of functionally important residues. These included the essential integrin binding sites (RGD and LDV) and residues involved in pentamerization and stable fiber-PB binding. Ad7PB was expressed as a fusion with *Schistosoma japonicum* glutathione S-transferase (GST) and was purified in a soluble form from *E. coli*. The fusion protein (GST–Ad7PB) and the cleaved (GST-free) Ad7PB both retained their ability to pentamerize as shown by electron microscopy. Although dodecahedra were not detected in the preparation, GST-Ad7 PB proved to be an effective and integrin-specific cell transfecting agent.

MATERIALS AND METHODS

Sequencing of *Ad7PB* gene

DNA coding for Ad7PB was amplified by PCR using total Ad7 viral DNA as template. PCR primers were designed on the basis of homology with Ad3PB and had the following sequence: Ad31 forward primer: AGCGGATCCAGTACGATGAGGAGACGAGCCGTG; Ad32 reverse primer, AGCAAGCTTTTAGAAAGTGCGGCTTGAAAGAA. The *Ad7PB* gene was cloned into pFastBac1 (Life Technologies, Bethesda, MD, USA) as a *Bam*HI–*Hin*dIII fragment to produce pAd7PB-FastBac1. The *Ad7PB* gene in pAd7PB-FastBac1 was subsequently sequenced by automated procedures.

Construction of expression plasmid pGST–Ad7PB

The *Ad7PB* gene was amplified by PCR from pAd7PB-FastBac1 and cloned as a *Bam*HI–*Eco*RI fragment in pGEX3X (glutathione S-transferase Gene Fusion system, Pharmacia). pGEX3X contains a factor Xa cleavage site between GST and PB to release GST and aid purification of the fusion partner. The primers used were: 7GX3UP forward primer: CTATGCGGGATCCCCATGAGGAGACGAGCCGTGCTA and 7GX3DN reverse primer: TGCTGCGAATTCTTCTTAGAAAGTGCGGCTTGAAAGAAC which incorporated, respectively, *Bam*HI and *Eco*RI cloning sites into the Ad7PB amplicon. The resulting construct, called pGST–Ad7PB, contained the Ad7PB open reading frame downstream of the *S. japonicum* GST gene. Expression from this vector is driven by the inducible Ptac promoter.

Expression and purification of recombinant GST–Ad7PB

The expression vector pGST–Ad7PB containing the GST–Ad7PB expression cassette was used to transform *E. coli* BL21 (λDE3). Transformed bacteria were grown in 1 L shake-flask cultures in the presence of 100 $\mu g \cdot mL^{-1}$ ampicillin to a $D_{600} = 1.2–1.5$ and induced with 0.1 mM (final) isopropyl thio-β-D-galactoside. The GST–Ad7PB fusion protein was purified by affinity chromatography on glutathione agarose (glutathione–Sepharose 4B, Pharmacia), according to the method of Rhim *et al* [27]. Briefly, induced total cell pellets from 1 L of culture were resuspended in 4 mL EBC buffer (50 mM Tris/HCl pH 8.0, 120 mM NaCl, 0.5% NP-40) containing 5 mM dithiothreitol. Lysates were prepared by homogenization (Polytron homogenizer, Kinematica GmbH) in the presence of 2 $mg \cdot mL^{-1}$ lysozyme (Sigma). Lysates were clarified by centrifugation at 12 000 *g* (SS-34 rotor, Sorvall) and supernatants were loaded on a 200-μL glutathione-Sepharose 4B column prewashed with EBC buffer. The column was washed with EBC buffer containing 200 mM NaCl to strip protein bound nonspecifically to the column. The fusion protein was eluted with glutathione elution buffer (10 mM GSH in 50 mM Tris/HCl pH 8.0). Two-hundred-microliter fractions were collected and analyzed on a reducing polyacrylamide gel. Protein concentrations were determined by the Bio-Rad Protein Assay reagent, and peak fractions were stored at 4 °C for further analysis.

Factor Xa-mediated cleavage of GST tag

The pGEX3X vector provides for a factor Xa cleavage site (Ile-Glu-Gly-Arg, with cleavage occurring after Arg) between GST and the fusion partner. Approximately 3 μg of affinity purified GST–Ad7PB were cleaved at room temperature for various time-points with 1 U of factor Xa (Sigma) in cleavage buffer consisting of 50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 1 mM $CaCl_2$. The cleavage reaction was terminated by addition of Laemmli buffer and analyzed on a 10% SDS/PAGE gel.

For EM analysis, GST–Ad7PB fusion protein was subjected to complete cleavage (verified by SDS/PAGE analysis), followed by GST–agarose separation to remove uncleaved material. Briefly, 10 μg of GST–Ad7PB were digested with factor Xa as described above. Digested reaction products were loaded onto a 200-μL glutathione–Sepharose 4B column prequilibrated with EBC buffer. The eluate was collected. The column was then washed with EBC buffer to elute any remaining cleaved Ad7PB, leaving any residual uncleaved GST–Ad7PB or GST adhering to the column. The eluate and wash-off fractions were then pooled, and concentrated with a Centricon-30 (Amicon) to a volume of 100 μL. The concentrated sample was subjected to EM analysis and to N-terminal sequencing (see below).

N-Terminal sequencing of Ad7PB

Factor Xa cleaved (GST-free) Ad7PB were blotted on to a PVDF membrane. N-Terminal peptide sequencing of the Ad7PB was performed by automated Edman degradation and HPLC using a Model 476A Protein Sequencer (Applied Biosystems).

Electron microscopy of GST–Ad 7PB and Ad7PB

GST–Ad7PB and factor Xa-cleaved GST–Ad7PB were dialyzed against water. Protein at a concentration of about 0.1 $mg \cdot mL^{-1}$ was adsorbed to the clean face of carbon on mica (the carbon–mica interface) and then the carbon film with adsorbed protein

was floated onto a solution of 1% sodium silicotungstate. A grid was placed on top of the carbon film which was then picked up from the top with a small piece of newspaper and air-dried. The samples were photographed in a JEOL 1200 EXII electron microscope at 100 kV under low-dose conditions at a nominal magnification of 40 000×.

For image analysis, two negatives of each sample were selected and digitized using an Optronics microdensitometer with a pixel size of 25 μm. We selected 1000 particles of Ad7PB and 600 particles of GST–Ad7PB using XIMDISP [28]. The particles were cut out of the field in squares of 64 × 64 pixels and filtered between 250 and 15 Å. All particles were centered using a circular object with the same diameter as the PB. Using the multivariate statistical analysis and the classification method implemented in SPIDER [29], all particles were classified into different subgroups ('class averages'). The Ad7PB images were very homogenous and 80% segregated into pentameric subgroups, the other 20% being side views or tilted views. The GST–Ad7PB group of images was much more variable, and only 50% of the particles segregated into pentameric subgroups.

Size analysis of GST–Ad7PB

To estimate the size of GST–Ad7PB, it was subjected to electrophoresis under nondenaturing and nonreducing conditions on a 8% separating gel with a 5% stacking gel. A number of high molecular mass proteins (Pharmacia) were included on the gel to provide size references. These were catalase (232 kDa), ferritin (440 kDa), thyroglobulin (660 kDa) and blue dextran (2 mDa).

DNA retardation by GST–Ad7PB

A gel-shift assay was performed to test whether GST–Ad7PB bound a bifunctional linker peptide derived from the adenovirus fiber protein. This peptide, designated FK20 [MTKRVRLSDSFNPVYPYEDEK(1–20)], was obtained by solid-phase synthesis, purified by RP-HPLC and stored dry at −20 °C. It contains the first N-terminal 20 amino acids of Ad7 fiber followed by 20 lysines. It binds PB protein due to the NPVYPY(12–17) sequence from Ad fiber [1,30] and is able to attach and compact DNA through the C-terminal polylysine domain. In addition, it contains the nuclear localization signal of the fiber protein, KRVR [30,31].

Complexes between GST–Ad7PB and FK20 were prepared by incubating GST–Ad7PB with FK20 at room temperature for 30 min. At the end of this time period, 1 μg of DNA was added to allow to complex via the C-terminal polylysine chain. Binding of plasmid by the GST–Ad7PB–FK20 complex was examined by analysis of the electrophoretic mobility of the plasmid DNA, using a 1% Tris/acetate/EDTA/agarose gel.

Ad7PB-mediated gene transfer

Adenovirus-susceptible human kidney epithelial cells (293 cells) were grown in DMEM containing 10% fetal bovine serum. Cells were maintained at 37 °C in the presence of 5% CO_2. GST–Ad7PB protein (5 and 10 μL corresponding to 1 and 2 μg of recombinant protein) was incubated with 1 μg of FK20 peptide for 30 min at room temperature. A reporter gene construct (pCMV-luc) containing the human cytomegalovirus immediate-early promoter cloned upstream of the firefly luciferase gene in plasmid pXP2 [32] was then added to the PB–FK20 linker complex, and the mixture was incubated for 30 min at room temperature (pCMV-luc was generated using standard recombinant DNA methods; not shown).

Cells were plated at a density of 2×10^5 per well in a 24 well plate, washed with serum-free medium and exposed to 100 μM chloroquine for 1 h. PB-DNA complexes were then added to cells in triplicate and incubated for 3 h at 37 °C in the presence of 5% CO_2. The PB–DNA complexes were removed at the end of 3 h, fresh complete medium was added, and the cells were incubated for an additional 60 h. At the end of this period, cells were washed with 1 × $NaCl/P_i$ and lysed in 100 μL reporter lysis buffer (Promega). Lysates were clarified by centrifugation at 5000 *g* and 20 μL of the clarified supernatants were assayed for luciferase activity using a microplate luminometer (LumiCount Model AL10000, Packard) in combination with the Luciferase Assay System (Promega). The results are expressed in relative light units (RLU) per microgram of cellular protein, as estimated using the Bio-Rad Protein Assay reagent.

RESULTS

Ad7PB gene sequence

Due to our interest in the use of the Ad3PB protein as a gene delivery system [15], we set out to examine whether the PB protein from a second, related strain of adenovirus might also have potential as a gene transfer vector. We therefore sequenced the gene coding for Ad7PB, because Ad7 is known to be closely related to Ad3, and also because Ad7 has previously been reported to have the ability to form dodecahedra, in a manner similar to Ad3 [26].

PCR amplification of the *Ad7PB* gene from Ad serotype 7 genomic DNA yielded a product of 1632 bp in length. The 544-amino-acid sequence of the *Ad7PB* gene was deduced from the nucleotide sequence and aligned with Ad2 and Ad3 PBs; Ad7 and Ad3 PB showed 99% identity at the amino-acid level (Genbank accession nos AAF37000and S41389, respectively), while Ad7 and Ad2 PB (Genbank accession P03276) were more divergent (77% predicted amino-acid identity). Both integrin-binding motifs, RGD (amino acids 329–331) and LDV (amino acids 296–298) were found to be conserved in Ad7PB. Also conserved was the RLSNLLG sequence (amino acids 263–269), identified as a putative fiber-binding domain on Ad2PB [1,33]. Sequences in Ad7PB flanking the RGD domain, a region known to exhibit considerable variation among Ad serotypes [34], were found to be almost identical to those found in Ad3PB (except for an asparagine residue at position 326 in Ad7PB vs. an aspartate at the corresponding location in Ad3 PB); in contrast, this region of Ad7PB is quite divergent from its counterpart in Ad2 (as previously noted by Karayan *et al* [34]).

Expression of Ad7PB in *E. coli* and purification by affinity chromatography

In order to further study the Ad7PB protein, we decided to express the molecule as a GST fusion protein in *E. coli*. We selected this expression system because it offers several advantages, including ease-of-use and detection, single-step purification and removal of the fusion partner (GST) using appropriate protease cleavage (e.g., factor Xa). In addition, the GST leader is believed to assist the fusion partner in folding and in its expression in the soluble form.

GST–Ad7PB was expressed in *E. coli* in amounts up to 5 $mg \cdot L^{-1}$ of culture. The fusion protein was soluble and was

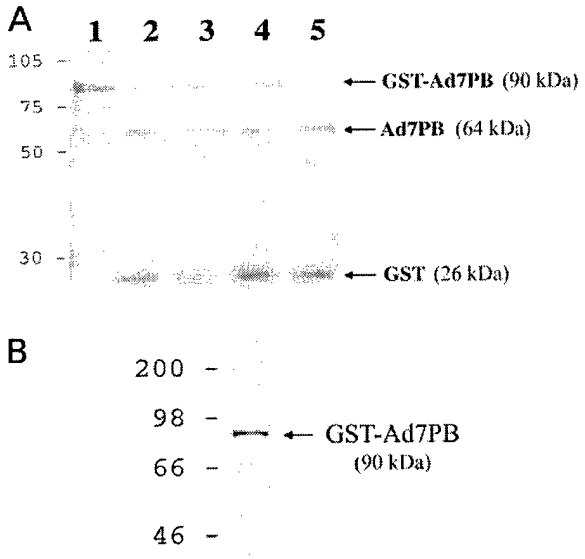

Fig. 1. Purification of GST–Ad7PB. (A) Factor Xa-mediated cleavage of GST–Ad7PB. GST–Ad7PB was incubated at room temperature with 1 U of factor Xa in 1 × cleavage buffer. Two-microgram equivalents of the protein were removed at various time-points and analyzed by electrophoresis on a 4–15% SDS-polyacrylamide gel (Bio-Rad). The gel was then stained with Coomassie brilliant blue. Uncleaved starting material (intact GST–Ad7PB, ≈90 kDa) is shown in lane 1, while cleaved Ad7PB (≈ 65 kDa) can be seen in the other lanes (lanes 2–5 represent 30, 45, 60 and 120 minute digestion with factor Xa). In all lanes, the presence of free GST protein (≈ 26 kDa) can observed; this is particularly pronounced after factor Xa cleavage. Numbers on the left represent molecular mass markers (figures in kDa). (B) Detection of GST–Ad7PB with an anti-Ad 3 PB Ig. Purified, full-length (not factor Xa-cleaved) recombinant GST–Ad7PB protein (2 μg) was electrophoresed on a 4–15% SDS-polyacrylamide gel (Bio-Rad), and an immunoblot was performed using a polyclonal rabbit antiserum directed against the adenovirus type 3 PB protein (this antiserum was generated in the Chroboczek lab., and used at a 1 : 100 000 dilution). Bound antibody was visualized using the ECL™ system (Amersham Pharmacia); numbers on the left represent molecular mass markers (figures in kDa).

purified from clarified bacterial lysates by single-step affinity chromatography on a glutathione–Sepharose 4B column. A 90-kDa band corresponding to the combined molecular masses of GST (26 kDa) and Ad7PB (64 kDa) was detected on a 4–15% SDS/PAGE (Fig. 1A, lane 1). This protein reacted with a polyclonal antiserum raised against the Ad3PB protein (Fig. 1B) and with an anti-GST Ig (data not shown), indicating that an authentic GST–Ad7PB protein was produced (note that Ad3PB is 99% identical to Ad7PB at the amino-acid level; thus a polyclonal antibody directed against Ad3PB can be expected to also recognize Ad7PB).

The GST–Ad7PB fusion protein could be cleaved with factor Xa to release the PB from the fusion protein. Factor Xa mediated cleavage of GST–Ad7PB at the Ile-Glu-Gly-Arg recognition site between GST and the beginning of Ad7PB yielded a protein of 64 kDa, corresponding to the expected molecular mass of the Ad7PB (Fig. 1A, lanes 2–5). N-Terminal peptide sequencing of the purified, factor Xa-cleaved Ad7PB protein confirmed that the purified protein did indeed correspond to Ad7PB (data not shown), and it also revealed the presence of copurified 60-kDa GroEL chaperone protein within the protein preparation (this is presumably present at low levels; Fig. 1A). However, total cleavage of the fusion protein could not be obtained within the 2-h reaction (see Fig. 1A). Thus, more complete digestion was required for studies in which GST-free Ad7PB was used (i.e. electron microscopy experiments; see below).

Structural characterization of Ad7PB proteins

Analysis of GST–Ad7PB by nondenaturing SDS/PAGE revealed that the recombinant protein migrated considerably more slowly than would have been expected for a GST–Ad7PB protein monomer (data not shown). This suggested that the recombinant GST–Ad7PB protein might have become organized into a pentameric configuration. Glutaraldehyde cross-linking and analysis by denaturing SDS/PAGE confirmed this possibility (data not shown), and we therefore undertook an EM analysis of both the recombinant GST–Ad7PB protein and also of the cleaved, GST-free Ad7PB protein.

Ad7PB and GST–Ad7PB were prepared for electron microscopy by negative staining. For EM analysis, GST–Ad7PB fusion was subjected to complete cleavage (verified by SDS/PAGE analysis), followed by GST–Sepharose separation to remove uncleaved material (see Materials and methods). Figure 2 shows pictures of fields of the two samples as well as selected untreated and averaged images in a gallery. The Ad7PB sample (Fig. 2A) contained homogeneous particles that could easily be classified in class averages that showed fivefold symmetry (e.g. Fig. 2A, bottom right) clearly showing the five subunits in the PB. This pentamer has a width of 100 Å the same as the width of the Ad3 PB [15]. The GST–Ad7PB sample was much less homogeneous because, although the PB part of the molecule was always the same and identical to the PB part in Ad7PB alone, the GST subunits seem to be rather flexibly linked to the PB, leading to a rather high number of class averages. Figure 2B shows a field of the sample and a gallery, together with a selected untreated image at the top and the class average it belongs to at the bottom. Because of the flexible PB–GST linkage, the class averages only clearly show two attached GST molecules at one time. The other three GST molecules are either not well embedded in the stain or not always located at exactly the same position compared to the other GST subunits, leading to loss of the signal in the average. From the class averages it can also be seen that the actual diameter of the GST–Ad7PB molecule is much higher than that of the PB alone and also much higher than the diameter of a globular complex with a corresponding mass. Neither the cleaved nor the uncleaved GST–Ad7PB formed dodecamers, as did the baculovirus produced Ad3PB [15]. However, the formation of pentamers by both Ad7PB and GST–Ad7PB indicates that pentamer formation by the *E. coli*-derived recombinant proteins is dependent upon the interaction of the PBs (i.e. pentamer formation is not dependent upon the presence of GST).

DNA retardation by GST–Ad7PB

GST–Ad7PB was effective in retarding the electrophoretic mobility of plasmid DNA, in the presence of the FK20 linker peptide, in a dose-dependent manner (Fig. 3). In the absence of the FK20 linker peptide, GST–Ad7PB alone failed to retard plasmid DNA (lanes 2–4). The migration pattern of pCMV-luc in these lanes was similar to the untreated plasmid in lane 1, which shows the mobility of free, supercoiled plasmid DNA. The binding and consequent retardation of pCMV-luc by the

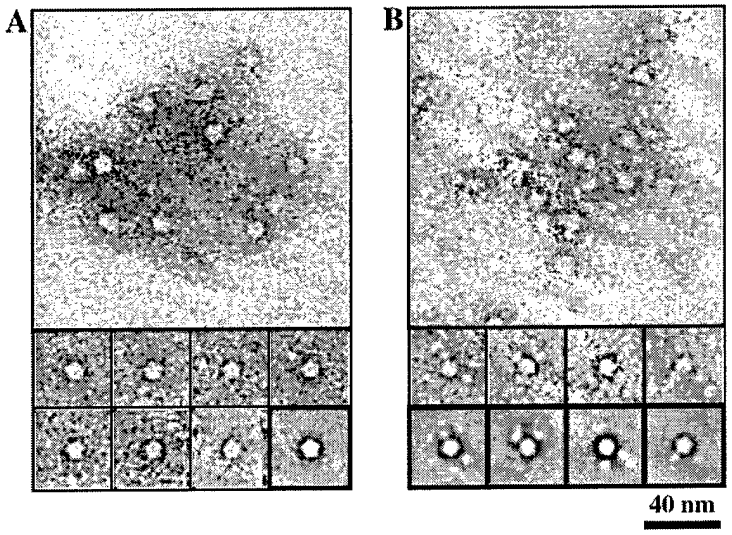

Fig. 2. Electron micrographs of negatively stained GST–Ad7PB and factor Xa cleaved Ad7PB. (A) Shows a field of factor Xa cleaved Ad7PB at the top and seven selected images of individual molecules. At the bottom right is a class average of 15 averaged raw images (see Materials and methods). (B) Shows a field of untreated GST–Ad7PB and four class averages calculated by summing 15 raw images. For each class average in the lower panel one of the raw images used to calculate the average is shown in the upper panel. The bar represents 40 nm (equals 400 Å).

GST–Ad7PB–FK20 complex is evident in lanes 5, 6 and 7 which contain increasing amounts (1, 2 and 4 μg, respectively) of GST–Ad7PB with a fixed amount of FK20 peptide. Plasmid DNA binding was dependent on Ad7PB and not on the GST moiety, as the mobility of plasmid DNA was unaltered in the presence of FK20 peptide plus GST alone (lanes 8, 9 and 10 which contain 1, 2 and 4 μg of GST, respectively).

GST–Ad7PB-mediated transfection

The ability of the GST–Ad7PB fusion protein to mediate gene transfer into cultured human cells was assessed by adding a complex of GST–Ad7PB plus FK20 preincubated with the luciferase reporter plasmid DNA (GST–Ad7PB–FK20–DNA) to 293 cells. Expression of luciferase was observed in 293 cells transfected with the GST–Ad7PB–FK20–DNA complex, either in the presence or absence of chloroquine (Fig. 4, lanes 4 and 5). Gene transfer by the GST–Ad7PB–FK20–DNA complex was somewhat less efficient in the presence of chloroquine (lane 5) than in its absence (lane 4). This may be due, in part, to

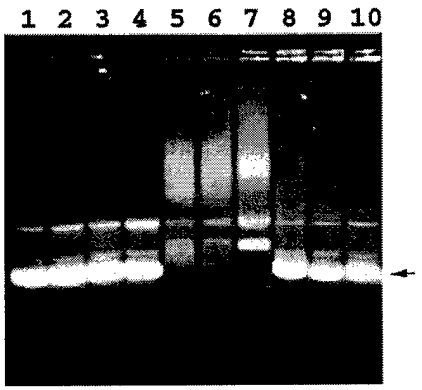

Fig. 3. GST–Ad7PB-mediated DNA retardation via FK20 linker. GST–Ad7PB or GST alone, was incubated with a luciferase reporter plasmid (pCMV-luc), in the presence and absence of 0.5 μg of the FK20 linker peptide. DNA–protein complexes were analysed by electrophoresis on a 1% agarose gel in 1 × Tris/acetate/EDTA. DNA was visualized using ethidium bromide and a photograph of the resulting gel is shown. Lane 1: untreated DNA incubated in buffer (this shows the mobility of free, supercoiled plasmid DNA; see arrow). Lanes 2, 3 and 4 contain plasmid DNA that was preincubated with 1, 2 and 4 μg of GST–Ad7PB (no FK20), while lanes 5, 6 and 7 contain DNA plus FK20 plus increasing amounts of GST–Ad7PB (1, 2 or 4 μg, respectively). Finally, lanes 8, 9 and 10 contain plasmid DNA plus GST (1, 2 or 4 μg, respectively) plus FK20.

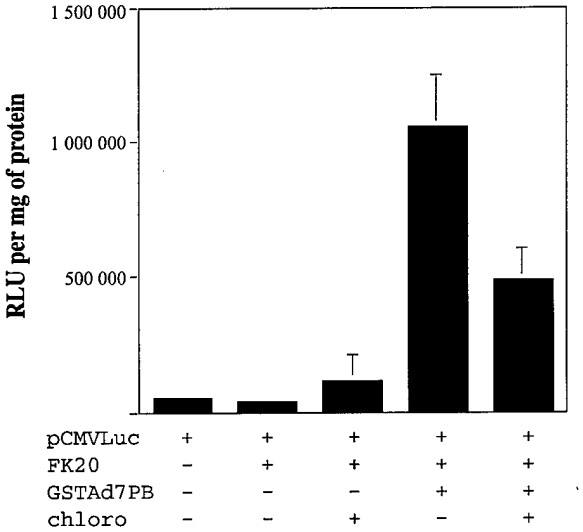

Fig. 4. GST–Ad7PB-mediated transfection of 293 cells. 293 cells were incubated with pCMV-luc and luciferase expression was measured in cell lysates collected 60 h after transfection. Lane 1, pCMV-luc added in the absence of GST–Ad7PB (control). Lanes 2 and 3, pCMV-luc and FK20 linker peptide added, in the absence (lane 2) or presence (lane 3) of chloroquine. Lanes 4 and 5, pCMV-luc plus FK20 plus GST–Ad7PB protein added, in the absence (lane 4) or presence (lane 5) of chloroquine. Luciferase activity is expressed in terms of relative light units (RLU) per mg of total protein in the cell lysates. Bars represent the standard error of mean values. The experiment was performed in triplicate and the results shown are representative of three experiments which yielded similar data.

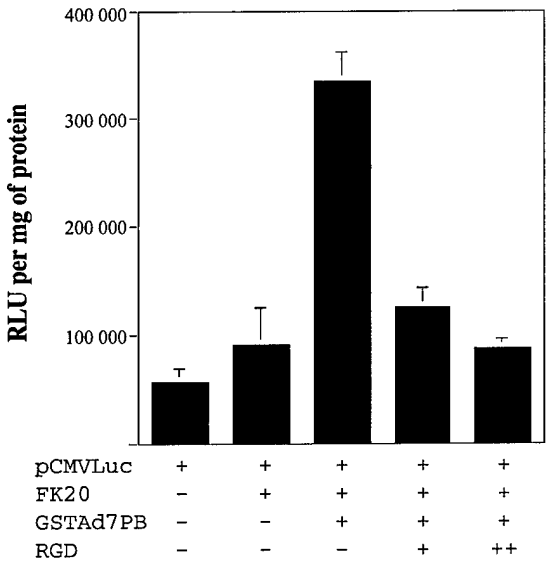

Fig. 5. Inhibition of GST–Ad7PB-mediated transfection of 293 cells by an RGD-containing peptide. 293 cells were incubated with pCMV-luc and luciferase expression was measured in cell lysates collected 60 h later. Lane 1, cells treated with pCMV-luc in the absence of GST–Ad7PB (control). Lane 2, Cells exposed to a complex of pCMV-luc and FK20 linker only. Lanes 3–5, Cells exposed to a complex of pCMV-luc and FK20 linker plus GST–Ad7PB protein, in the absence (lane 3) or presence (lanes 4 and 5) of an RGD-containing peptide (NITRGDTYI; added at 10 or 100 μg to lanes 4 and 5, respectively). Luciferase activity is expressed in terms of relative light units (RLU) per mg of total protein in the cell lysates. Bars represent the standard error of mean values. The experiment was performed in triplicate and the results shown are representative of three experiments which yielded similar data.

possible toxic effects of chloroquine under the conditions used. In any event, a much lower level of luciferase activity was detectable in cells transfected with complexes containing FK20 peptide plus DNA (i.e. complexes lacking the GST–Ad7PB protein). This is consistent with our previous finding that an analogous peptide derived from the adenovirus type 2 fiber protein can mediate DNA transfer into some human cell types [35].

While Ad7-derived FK20 peptide could mediate a low level of DNA uptake into 293 cells, perhaps due to the presence of a tyrosine-based motif (NPXY) that may function as an internalization signal involved in receptor-mediated endocytosis [36–38], DNA delivery was much more efficient when the GST–Ad7PB protein was added to the transfection complex, and transfection also occurred in the absence of chloroquine (Fig. 4).

To investigate the specificity of DNA transfection by the GST–Ad7PB–FK20–DNA complex, cells were preincubated in the presence of an excess of an RGD-containing peptide derived from the Ad7PB (NITRGDTYI) prior to the addition of the GST–Ad7PB–FK20–DNA complex. This resulted in a strong dose-dependent inhibition of luciferase expression in the transfected cells (Fig. 5, lanes 3–5), suggesting that the GST–Ad7PB protein facilitates DNA uptake via an integrin-dependent pathway.

DISCUSSION

The adenovirus PB may be an ideal molecule to exploit as a gene transport vehicle, because it is able to interact in a selective manner with cell-surface integrins to effect internalization. We sequenced the full-length gene coding for Ad7PB protein and found it to be 99% identical to the Ad3PB at the amino-acid level. Ad7PB was also found to retain the integrin-binding peptide motifs present in PB proteins from several other adenovirus serotypes (RGD and LDV), as well as residues involved in penton-fiber binding and pentamerization factors that were critical for DNA delivery.

Expression of Ad7PB as a C-terminal fusion with *S. japonicum* GST in *E. coli* BL21 cells yielded soluble protein that could be purified from bacterial lysates by a single-step affinity chromatography. To our knowledge, this is the first report of the production of stable adenoviral pentons from *E. coli*. Bai *et al* [6,14]. have reported bacterial expression of Ad2PB and Ad12PB. In both cases, the PB was found to localize in *E. coli* as insoluble inclusion bodies. Our method represents an improvement over the method reported by Bai and coworkers, both in the soluble nature of expression and also in terms of ease of down-stream processing.

Structural characterization of Ad7PB by negative stain electron microscopy revealed that the *E. coli*-derived protein was pentameric, like the native form of this protein. Although the PBs formed from cleaved, GST-free, Ad7PB were more symmetrical, the GST-tagged protein retained the inherent spontaneous capability of forming pentamers. The fact that a 26-kDa addition (GST) at the N-terminus of PB did not inhibit pentamer formation indicates that signals for pentamerization may be located distal to the PB N-terminus. Indeed, most of the residues implicated so far in pentamerization are present at the C-terminus of PB (Y553 and K556) or are located far from the N-terminus (W119) [33]. In addition, the ability of GST–Ad7PB to bind a fiber-like peptide (FK20), as reflected by the results of our DNA retardation experiments (Fig. 3), suggests that the presence of the GST moiety does not interfere with fiber-binding of Ad7PB. Thus, the conformation of the GST–Ad7PB fusion protein appears to be biochemically indistinguishable from that of its native (unfused) counterpart.

N-Terminal sequencing of GST–Ad7PB also revealed an association of the 60-kDa chaperonin GroEL with GST–Ad7PB. *E. coli* GroEL is known to bind a number of proteins expressed as GST fusions in bacteria [5,39] and to aid the formation of native structures. Battistoni *et al* [39]. have demonstrated that *E. coli* chaperonins are able to interact with nascent GST. It is possible that GroEL plays an analogous role in the folding of GST–Ad7PB and assists in the formation of pentamers.

Gene transfer efficiency by the GST–Ad7PB–FK20–DNA complex was found to exhibit significant variation in different experiments. For example, the efficiency of gene expression in Fig. 4, lane 4 is roughly threefold greater than that in Fig. 5, lane 3, even though the cells in these experiments were exposed to the same reagent (GST–Ad7PB + FK20 + pCMV-luc), in the absence of chloroquine. We attribute this variation to the fact that the large macromolecular complex represented by GST–Ad7PB–FK20–DNA is held together by at least two separate interactions: a protein–peptide interaction between GST–Ad7PB and FK20, and an electrostatic interaction between FK20 and DNA. The latter interaction is likely to be particularly susceptible to slight differences in storage conditions and ionic strength of any buffers used. Slight fluctuations in pH may also alter the strength of the various interactions which hold the GST–Ad7PB–FK20–DNA complex together.

Nonetheless, GST–Ad7PB was found to transfect 293 cells in an integrin-dependent fashion (as expected). Furthermore, GST–Ad7PB-mediated gene transfer was not improved by the

presence of chloroquine (indeed, it was enhanced in the absence of the drug; Fig. 4, lanes 4 and 5). This suggests that GST–Ad7PB-mediated gene transfer occurs either by a lysosome-independent pathway, as described for fibroblast growth factor and HIV-1 Vpr [40,41], or as a consequence of PB-mediated endosome escape in 293 cells [42].

In previous experiments using recombinant Ad3PB dodecahedra prepared from insect cell lysates, we obtained levels of reporter gene expression in transfected 293 cells that were approximately 1000-fold higher than the levels reported here, using the *E. coli*-derived GST–Ad7PB fusion protein [15]. Similarly, the level of transfection obtained with commercial transfection reagents such as lipofectamine or DOTAP was 10–20 times higher than the level reported here.

The difference in transfection efficiency between the Ad3PB dodecahedra and the recombinant GST–Ad7PB protein preparations used here cannot be attributed simply to the presence of the GST moiety, as factor Xa-cleaved GST-free Ad7PB protein mediated a similar level of DNA transfer to that obtained with the GST–Ad7PB fusion (data not shown). Thus, the differences between our current findings and those reported with the insect cell-derived Ad3 base dodecahedra preparations may be due to inherent differences in structural features of the pentameric PB vs. dodecahedra. GST–Ad7PB assumes a pentameric configuration that possesses a total of five RGD motifs (integrin-binding domains) and five binding sites for the FK20 linker peptide. This may explain why base dodecahedra (60 RGD motifs and 60 binding sites for the FK20 peptide) are more efficient gene delivery systems than PB [15]. We are currently devising strategies to modify *E. coli*-expressed PB in such a manner as to facilitate the formation of dodecahedra.

Overall, the results reported here establish the potential utility of *E. coli*-derived Ad7PB as a gene delivery agent. Further experiments, including mutagenesis of the residues flanking the RGD motif, will be required in order to optimize this technology, and to exploit its potential for integrin-mediated gene delivery to specific target cell populations [43].

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